Impedance Measurements Could Accelerate Phage-Based Identification of *Bacillus anthracis* And Other Bacteria

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The high degree of specificity that phage exhibit for their hosts has made them valuable tools for identifying bacterial species and subspecies. This is particularly true in the field of biodefense where phage have a long history of being used to identify *Bacillus anthracis* and *Yersinia pestis* isolates. However, phage assays generally require that suspect colonies be sub-cultured onto a fresh agar plate to generate a dense lawn against which the phage-induced plaques can be observed. Following a potential exposure to bacterial threat agents, this additional incubation step can consume valuable time. Researchers have shown that temporal changes in the dielectric permittivity of bacterial micro-cultures differ for chemically or physically stressed and unstressed cells. In fact, a distinctive shift in the relative responses caused by heat shock, antibiotics, or phage infection can be detected as early as one hour after exposing as few as 10^5 CFU bacteria to the stressor. We predicted that similar responses could be used to detect ɣ phage-induced stress in susceptible *B. anthracis* micro-cultures and thereby reduce both the time and biomass required to perform phage-based diagnostic assays for this pathogen. After exposing small quantities of *Bacillus* cultures to ɣ phage, we tracked the cultures for up to 90 minutes using microscopy, impedance measurements, and plated serial dilutions. Our results show that only the ɣ phage-sensitive *B. anthracis* cultures exhibit rapid and distinctive drops in dielectric permittivity when exposed to the phage. This data suggests that impedance measurements might be used to improve traditional phage-based identification assays, including those used in the field of biodefense.

Background

There is a long history of successfully using highly specific bacteriophage to positively identify bacterial isolates in clinical and environmental samples. As is the case with the *Bacillus anthracis* ɣ phage assay [1], the ability of a known phage suspension to form plaques on a lawn of unknown bacterial isolate is taken as proof that the sample is the cognate host of the phage.

Despite their relative simplicity, a potential drawback of traditional plaque assays is that they require time for the plated bacteria to form confluent growth and for phage-induced plaques to appear. Techniques that permit faster detection of species-specific bacteria/phage interactions would increase the value of the phage-sensitivity assays in clinical settings.

There is already a growing body of literature, which shows that prokaryotes produce detectable stress responses within minutes of being exposed to a range of specific and non-specific stresses such as antibiotics, acids, peroxides, and heat [2-5]. Similar responses likely occur soon after bacteria come in contact with their cognate phage.

Along these lines, BioSense Technologies, Inc., has reported that chemical and heat-induced stress responses can be rapidly detected as changes in the dielectric permittivity of bacterial micro-cultures containing as few as 10^5 colony forming units [6, 7]. The group has also accumulated unpublished results showing that the dielectric permittivities of *Escherichia coli*, *Mycobacterium smegmatis*, and *Bacillus subtilis* cultures are significantly altered by the presence of their cognate phage as soon as one hour after exposure. These observations indicate that changes in capacitance across bacterial cultures could offer early signs of microbial sensitivity to species-specific phage.

In order to further assess whether impedance measurements can be used to rapidly detect phage sensitivities in clinical and environmental samples, we studied the responses of closely-related *Bacillus anthracis*, *cereus,* and *subtilis* isolates to ɣ phage. Since ɣ phage generally only infects *B. anthracis* species, the phage is frequently used to distinguish the bio threat agent from other less virulent *Bacilli*.

Our study reveals that *B. anthracis* micro-cultures uniquely exhibit detectable shifts in their dielectric permittivities as soon as 90 minutes after exposure to ɣ phage and well before bacterial growth is adversely affected by the phage infection. These findings further underscore the role impedance assays can play in supporting rapid clinical and environmental diagnostics.

Materials and Methods

Phage and spore preparations. ɣ phage were propagated by infecting the lawns of *B. anthracis* CDC 684 vegetative cells on sheep's blood agar (SBA) and then incubated for approximately 18 h at 35°C. The phage were harvested by scraping off plaque rich areas of lawn then collecting the gathered material with a Nutrient Broth (NB) rinse. Bacterial cells were removed by centrifuging for 10 min at 10,000 x g. The gamma phage rich supernatant was then run through a 0.2 µm filter and sterility checked by plating 10% on SBA. After using a standard plaque assay to measure amount of phage in the supernatant, the concentration was adjusted to 3.7 x 10^9 PFU/mL by further diluting the suspension into NB.

Bacillus spores were produced by plating vegetative cells in lawn onto New Sporulation Medium (NSM), which consisted of 3 g Tryptone, 3 g Yeast Extract, 2 g Agar, 23 g Lab Lemco agar, and 1 mL 1% $MnCl_2 \cdot 4H_2O$ in 1 L dH_2O. The *Bacillus*-inoculated NSM agar plates were incubated at 35°C for at least 48 h until Gram stains revealed the presence of > 90% *Bacillus* spores in the plated colonies. The cells were then resuspended in sterile dH_2O and maintained at 4°C for at least one week until vegetative cells were no longer visible in Gram stained samples. Finally, centrifugation was used to remove soluble debris from the preparation and spore concentrations were assessed by dilution plating on SBA.

Bacillus spores were prepared from *B. anthracis* Sterne, *B. cereus* BCTC 2599, *B. anthracis* ΔANR (BACI104), *B. anthracis* STI (BACI004), and *B. subtilis* NRS231 (BACI033) reference material lots, obtained from the US Department of Defense's ISO accredited Unified Culture Collection (http://www.usamriid.army.mil/ucc/).

Plaque assays. Plaque assays were performed by suspending 10^7 CFU *Bacillus* spores in 100 µL Tryptic Soy Broth (TSB) and spreading on both hemispheres of a SBA plate using a 10 µL inoculating loop. 10 µL aliquots of viable or heat-inactivated ɣ phage were spotted onto each hemisphere in three locations. Heat-inactivated phage were prepared by heating the phage suspension for 15 minutes at 75°C and cooling it on ice prior to use. The phage-treated agar plates were incubated overnight at 35°C before examining them for the presence or absence of plaques.

Bacterial cultures and phage infections. Bacterial cultures were prepared by inoculating 1 mL TSB (Remel) with 10^7 CFU *Bacillus* spores and incubating the cultures for 60 minutes at 35°C. In cases where the bacteria were exposed to phage, the hour-old cultures were mixed 1:1 with 3.7×10^9 PFU/mL viable or heat-killed ɣ phage in NB. Heat-inactivated phage were prepared as described above by heating the phage suspension for 15 minutes at 75°C and cooling on ice prior to combining with the bacterial culture. Phage-treated bacterial cultures were observed for 90 minutes post treatment.

Gram Stains. Gram stains were used to track the emergence of vegetative *Bacillus* cells from spores. In this assay, bacterial cultures were prepared as described above and incubated for 90 minutes at 35°C without being exposed to ɣ phage. At 30, 60, and 90 minutes post inoculation, we spotted 10 µL culture aliquots onto microscope slides and allowed them to air dry. Cells were fixed to the slide using methanol and sequentially stained with Gram Crystal Violet, Gram Iodine, Gram Decolorizer, and Gram Safranin following the manufacture's protocol (Remel). The cells were viewed and photographed with an Olympus (Center Valley, PA) BX41 microscope and DP72 digital camera.

Electron microscopy. Fifteen minutes after mixing hour-old bacterial cultures with viable phage as described above, a 100 µL aliquot was adsorbed to a 0.2 µm filter and fixed at 4°C in a solution of 2% glutaraldehyde, 4% paraformaldehyde, and 0.1 M phosphate buffer. The fixed filters were dehydrated, critical point dried, and coated with carbon. Samples were imaged in a Zeiss (Thornwood, NY) Sigma Variable Pressure Field Emission Scanning Electron Microscope (FE-SEM) in variable pressure mode with ~25 Pa of pressure at 5 kV.

Colony counts. In order to assess the short-term effects of phage infection on bacterial growth, colony counts were prepared from bacterial cultures immediately after being mixed with viable or heat-

inactivated phage and then again after 90 minutes. These counts were obtained by spreading 100 µL aliquots of 10-fold dilutions on SBA and incubating overnight at 35°C. Each of the serial dilutions were performed three or more times to obtain the reported results.

Impedance measurements. Immediately after mixing an hour-old bacterial culture with a viable or heat-killed phage solution (see above), a 100 µL aliquot of the mixture was loaded into a chamber B of the Z-Sense cassette (BioSense Technologies, Inc., Woburn, MA). A sterile 1:1 mixture of TSB and NB was loaded into chamber A to provide a corresponding baseline measurement. The cassette was placed in the Z-Sense impedance meter (BioSense Technologies, Inc.) and allowed to sit at 37°C for 20 minutes before monitoring the normalized impedance response (NIR) between the two chambers for another 70 minutes. The NIR was computed by dividing the capacitance across chamber B by the capacitance across chamber A and normalizing the result against the value at the 20-minute mark. All cassettes were cleaned and tested daily before use to confirm that the NIR did not deviate from 1.000 by more than 0.002 when sterile media was loaded into both chambers and monitored for 90 minutes. Reported results aggregate three or more independent experiments conducted on separate days.

In a variant of the above assay, two aliquots of the same hour-old bacterial culture were mixed 1:1 with 3.7×10^9 PFU/mL heat-killed or viable ɣ phage and loaded into chambers A and B, respectively, of the Z-Sense cassette.

Results

Viable ɣ phage rapidly affect the dielectric permittivity of *B. anthracis* micro cultures

We tested the effects of viable and heat-inactivated ɣ phage on the dielectric permittivity of *B. anthracis* Sterne *and B. cereus* NCTC 2599 cultures using BioSense Technologies' Z-Sense (**Figure 1**), which works by comparing the capacitance across the two 100 µL chambers that are maintained at an internally monitored 37°C.

The Z-Sense reports the NIR, which is computed by dividing the capacitance of chamber B with that of chamber A and multiplying by a constant that scales the NIR to 1 at the start of the assay. This calculation allows us to track the performance of the test culture in chamber B as it relates to the control culture in chamber A. Through repeated experimentation we have found that it takes about 20 minutes for the measurements across both chambers to equilibrate. Consequently, all measurements reported in this paper start 20 minutes post challenge and the NIR is normalized to this time point.

We began each assay by inoculating 1 mL TSB with 10^7 CFU *Bacillus* spores and incubating the cultures at 35°C for 60 minutes without shaking. As shown in **Figure 2**, we used Gram stain to confirm that the 60 minute incubation time allows the *Bacillus* spores to germinate without dividing. This technique of preparing vegetative cells directly from spores allowed us to carefully control the bacteria/phage multiplicity of infection (MOI) from one experiment to the next. It also ensured that we exposed the cells to ɣ phage before they produced sufficient capsule to inhibit phage infection.

At the end of the one hour incubation, the vegetative *Bacillus* culture was combined with an equal volume of 3.7 x 10^9 PFU/mL live or heat inactivated ɣ phage and immediately loaded into chamber B of the Z-Sense cassette. Chamber A was loaded with a sterile media control. In an earlier experiment we had assayed the activities of viable and heat-inactivated phage by spotting the phage on confluent lawns of the *B. anthracis* Sterne and *B. cereus* NCTC 2599. These results, which are shown in **Figure 3**, confirmed that sensitivity of *B. anthracis* Sterne to the phage and the effectiveness of our heat inactivation procedure.

Figure 4 displays the averaged NIR values obtained from for four sets of replica assays ($n \geq 4$) in which the chamber B was loaded with vegetative *B. anthracis* Sterne or *B. cereus* NCTC 2599 cultures immediately after being exposed to viable or heat inactivated ɣ phage. These results show that adding viable phage to the *B. anthracis* culture significantly depresses the NIR over the course of the 90 minute assay when compared to the effects of the heat inactivated phage. In contrast, adding viable phage to ɣ phage-insensitive *B. cereus* cultures has no discernable effect on the NIR.

ɣ phage do not impact cell growth during the course of the impedance assay

In order to better understand what is happening to the *Bacillus* cultures during the course of the 90-minute impedance assay we used scanning electron microscopy and dilution plating assays to look for evidence of phage binding and growth inhibition in the treated cultures.

We probed the specificity and kinetics of ɣ phage binding by adding viable phage to *Bacillus* cultures and passing the solution through a 0.2 µm filter 15 minutes after exposure to remove any unbound phage. We then fixed the cells to the filter and observed them under an electron microscope. As shown by the examples in **Figure 5**, electron micrographs consistently revealed phage bound to the *B. anthracis* Sterne bacteria but not the flagellated *B. cereus* NCTC 2599 cells. This confirms that ɣ phage specifically bind *B. anthracis* and that this interaction occurs very early in the course of the impedance assay, when capsule does not prevent the phage from attaching to the cell.

We also considered whether the observed impedance effects were the direct result of a phage-induced drop in viable cell counts. We investigated this by preparing *Bacillus* cultures as described previously. However, immediately after mixing aliquots of each culture with viable and heat-inactivated phage, as we had done for the above impedance assay, we plated a fraction of each suspension on SBA and counted colonies after approximately 16 h at 35°C. We similarly plated aliquots of the same phage-treated cultures after they had been maintained at 37°C for 90 minutes. Our results, which were performed in triplicate for both *B. anthracis* Sterne and *B. cereus* NCTC 2599, are graphed in **Figure 6** and show no significant change in CFUs during the course of the 90 minute impedance assay.

Impedance can be used to quickly assess ɣ phage sensitivities in *Bacillus* micro cultures

The preceding assays suggest that impedance can be used as a rapid diagnostic indicator of highly specific bacteria/phage interactions. However, the process can be streamlined further by directly comparing the dielectric permittivities of cultures containing bacteria with viable ɣ phage to those containing the same bacteria with heat inactivated phage. In this assay, the NIR of bacterial cultures that are unaffected by the presence of viable phage will not be deflected from the normal. On the other

hand, when ɣ phage-sensitive *B. anthracis* are present, the NIR should trend downward as a result of capacitance differences across the test chamber, which contains bacteria with viable phage, and the control chamber, which holds bacteria with inactivated phage.

Under the revised protocol, TSB was inoculated with *Bacillus* spores and incubated at 35°C for 60 min. The cultures were then split and mixed with viable or heat-inactivated phage before loading into the test or control chambers of the Z-Sense cassette. As in the previous assay, the NIR was monitored for 90 minutes post challenge.

We performed this assay on *B. anthracis* Sterne, *B. cereus* NCTC 2599 and three other *Bacillus* strains as a means of demonstrating applicability to a broader range of ɣ phage sensitive and insensitive bacteria. The average NIR values for replica assays ($n \geq 3$) are shown in **Figure 7** where they are compared against NIR values for control runs in which the bacteria were absent. As expected, our results show a significant deflection below the normal for each of the cultures containing ɣ phage-sensitive *B. anthracis* strains. Conversely, the ɣ phage-insensitive *B. cereus* and *B. subtilis* cultures produce NIRs which fail to deviate significantly from the normal during the 90 minute assay. In each case bacterial sensitivities to viable and heat inactivated ɣ phage were confirmed using a standard plaque assay and shown to be consistent with the outcomes of the impedance assays.

Conclusions

We were inspired to conduct this study after learning of unpublished research by Rieder and Zhao, which shows that the dielectric permittivities of *Escherichia coli*, *Mycobacterium smegmatis*, and *B. subtilis* cultures are altered by the presence of their cognate phage as soon as 60 minutes after exposure. Our own investigation suggests that impedance measurements can be used to similarly detect ɣ phage-sensitivities in *Bacillus* cultures after as little as 90 minutes. In fact, this distinctive shift in dielectric permittivity for phage-sensitive cultures occur shortly after ɣ phage attach to the bacteria and well before the infection adversely affects bacterial growth. Consequently, the phage impedance assay described above have the potential to greatly reduce the time required to identify *B. anthracis* isolates based on their unique physiological responses to the highly specific ɣ phage.

Zavizion, et al. have reported that the same mechanism can be used to support the identification of antibiotic sensitivities [7]. Given the small number of cells required for each impedance assay, it is conceivable that this technology can support rapid multiplexed phage and antibiotic sensitivity screens on small quantities of cells. Looking ahead, this could make the impedance-based detection of bacterial stress responses an appealing technology for clinical and environmental diagnostic applications.

Acknowledgements

We would like to thank Ron Rieder and Zhihui Zhao of BioSense Technologies, Inc., for their help and guidance at the outset of this study. We are also grateful to Sanjay Krishnaswamy for suggesting the potential application of impedance measurements to phage-sensitivity assays. This study was made possible, in part, by support from the Critical Reagents Program under the United States Army's Joint

Program Executive Office for Chemical and Biological Defense. Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the United States Army.

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Figures

A. B.

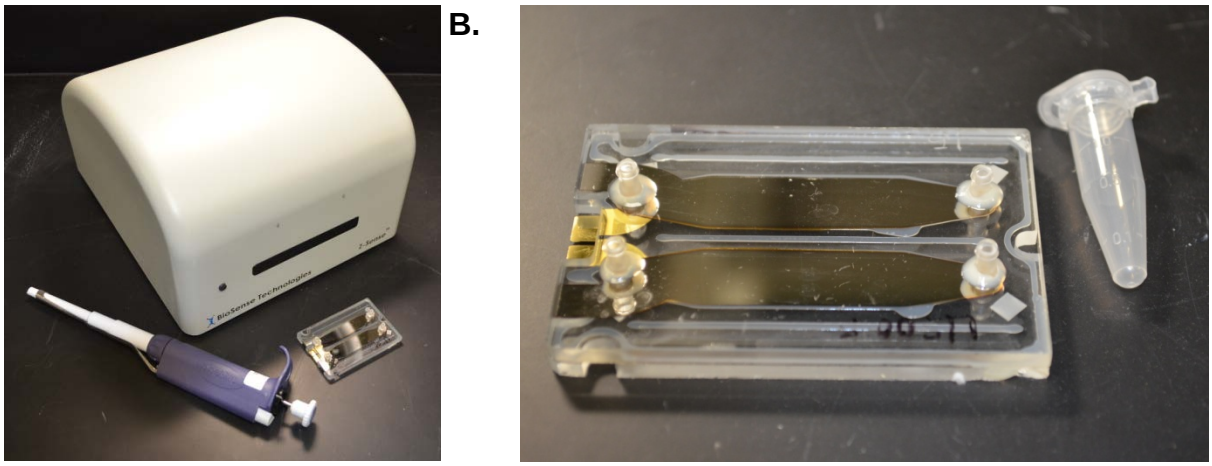

Figure 1. Impedance can be used to distinguish stressed and unstressed bacterial cultures. BioSense Technologies, Inc. manufactures the Z-Sense impedance meter (A), which measures the differential capacitances across two 100 µL cultures contained in a reusable microcassette (B). Previous studies have shown that when bacterial cultures are placed in one chamber of the cassette and sterile media is added to the other, the NIR between the two chambers either raises or drops depending on whether the cultures are physically stressed [6, 7].

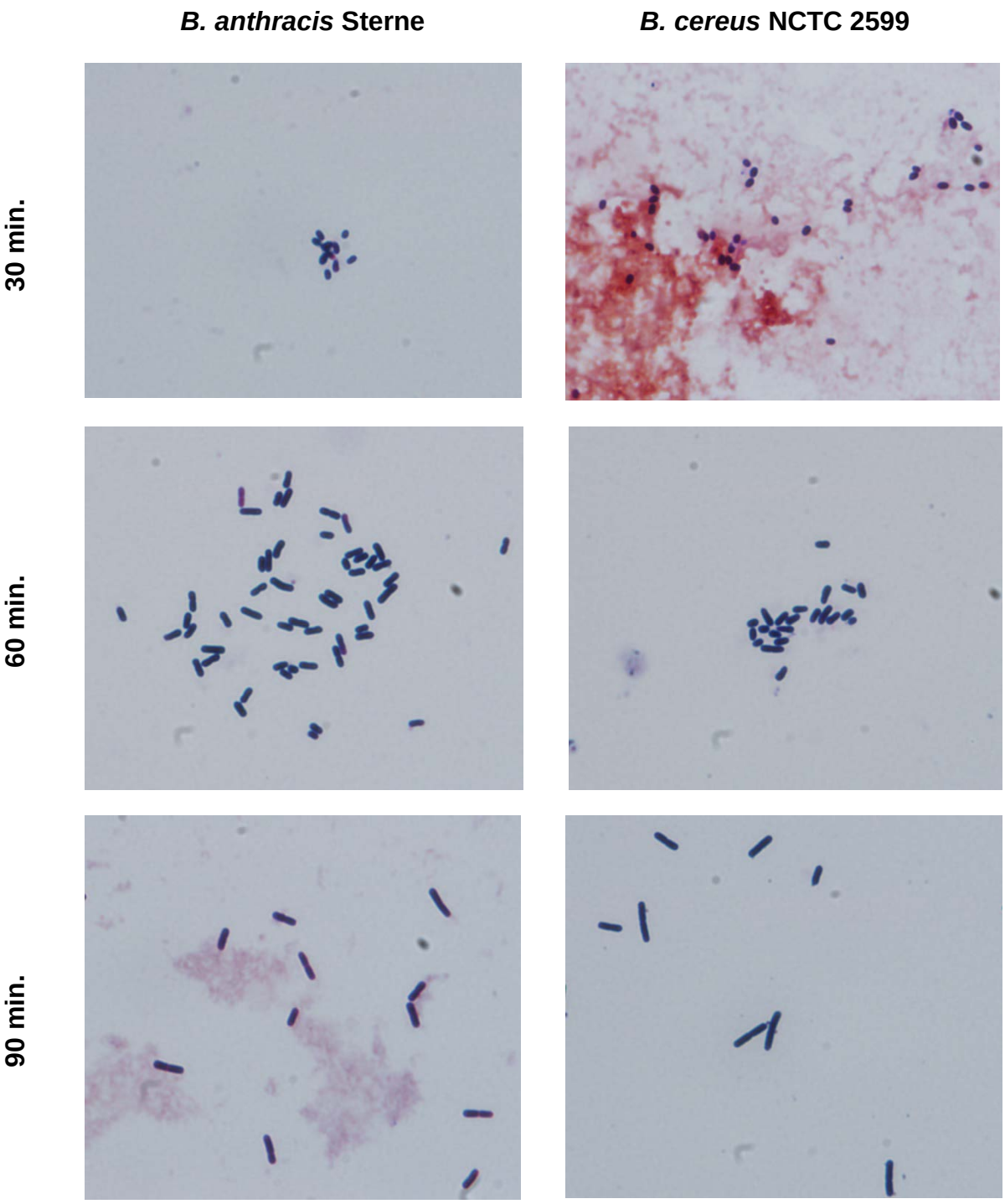

Figure 2. Most *Bacillus* spores have entered a vegetative state 60 minutes after inoculation. Gram stains reveal that *B. anthracis* Sterne and *B. cereus* NCTC 2599 cultures similarly progress from spores to vegetative bacteria at 60 minutes and replicating cells at 90 minutes. In our impedance assay, cultures are treated with phage at the 60 minute time-point when the bacteria have germinated but not yet undergone replication.

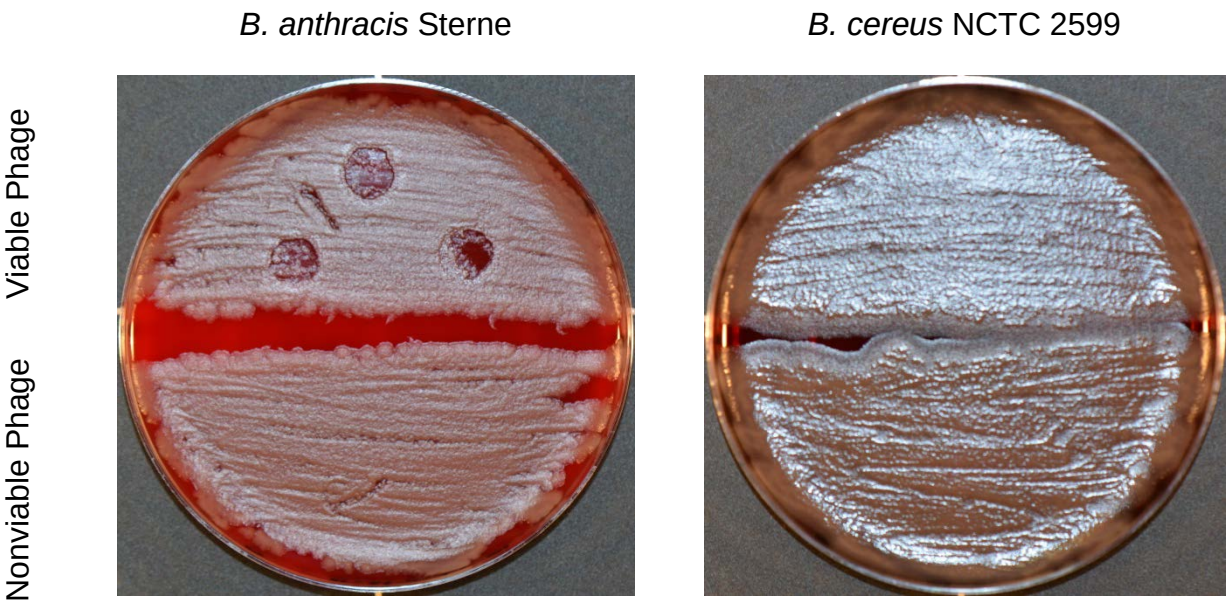

Figure 3. Viable ɣ phage specifically infect *Bacillus anthracis*. When *B. anthracis* Sterne or *B. cereus* NCTC 2599 spores were spread on sheep's blood agar and spotted with 10 µL 10^9 PFU/mL viable or heat-inactivated ɣ phage, only the viable phage formed plaques where applied to the *B. anthracis* lawn. The high specificity of bacterial phage is frequently exploited by clinicians to help identify cognate bacteria in clinical and environmental samples.

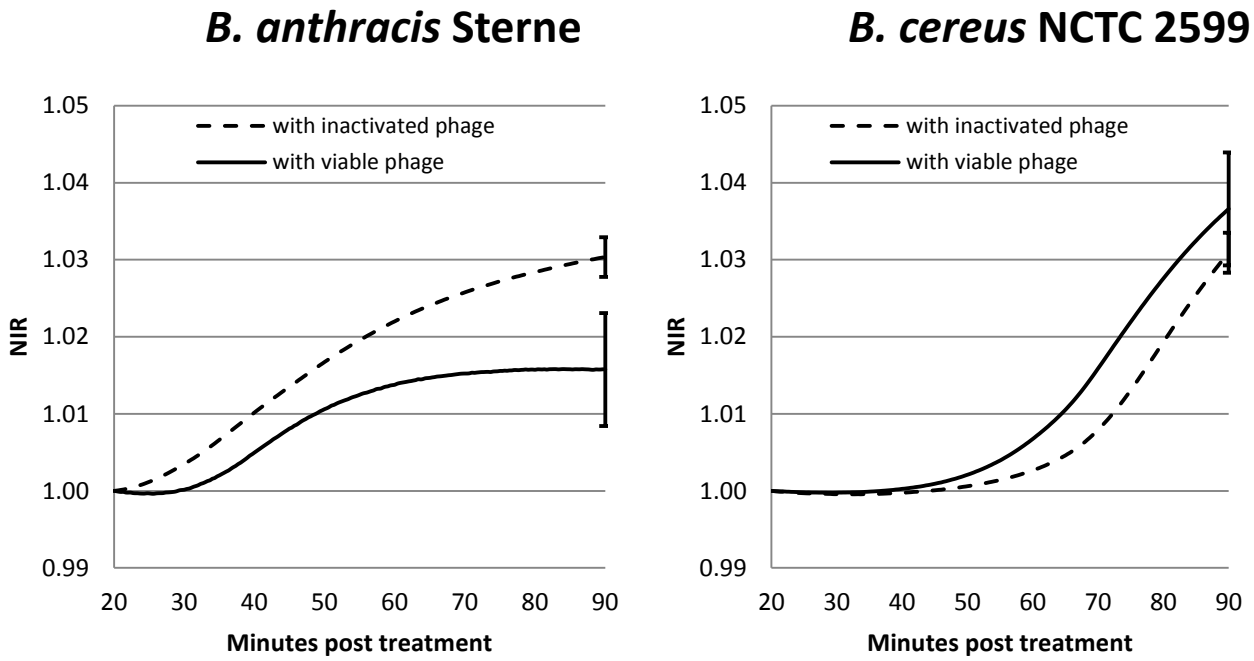

Figure 4. Viable ɣ phage significantly impact the normalized impedance response of *B. anthracis* cultures. The NIR of *B. anthracis* Sterne cultures are significantly suppressed 90 minutes after exposure to viable ɣ phage when compared to cultures similarly treated with heat-inactivated phage. The NIR of *B. cereus* NCTC 2599 cultures do not exhibit the same sensitivity to viable ɣ phage. These results suggest that impedance measurements allow detection of phage sensitivities before infections adversely impact affected cell populations. The NIR was calculated by dividing the capacitance of the chamber containing bacteria and viable or inactivated phage with that containing only media. Error bars depict the standard deviations obtained from four or more independent experiments.

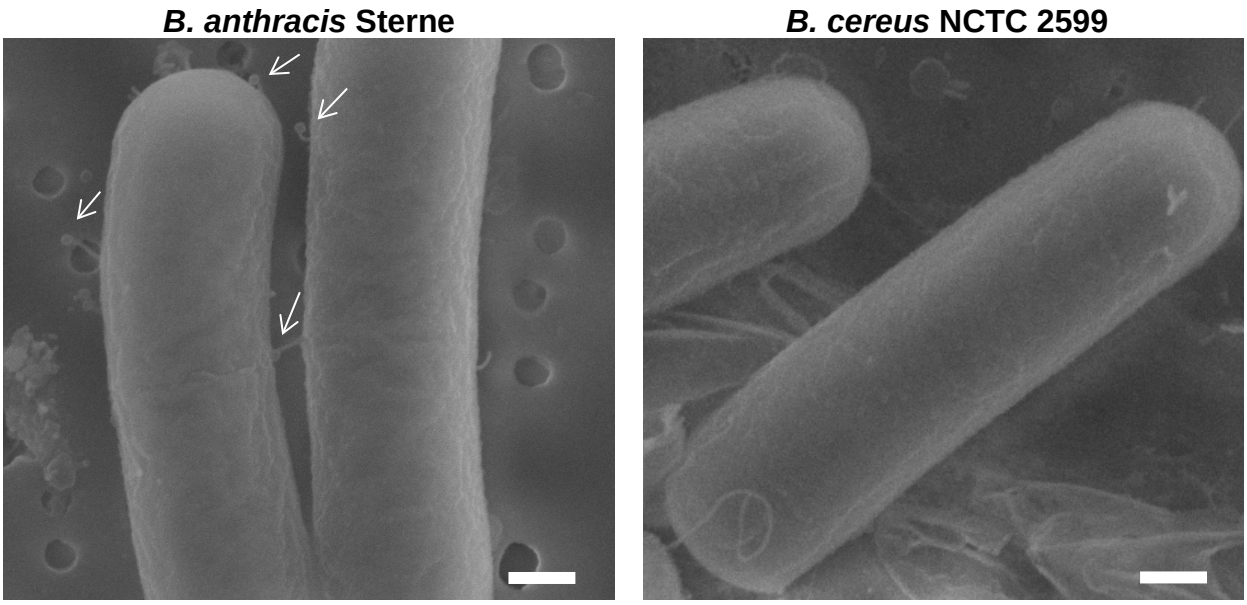

Figure 5. Ɣ phage specifically bind *B. anthracis* shortly after exposure. Electron microscopy shows ɣ phage (indicated by white arrows) attached to *B. anthracis* Sterne cells 15 minutes post exposure. Similar interactions were not observed for ɣ phage-treated *B. cereus* NCTC 2599 cells, also shown here. A white 300 nm scale bar is included in each image.

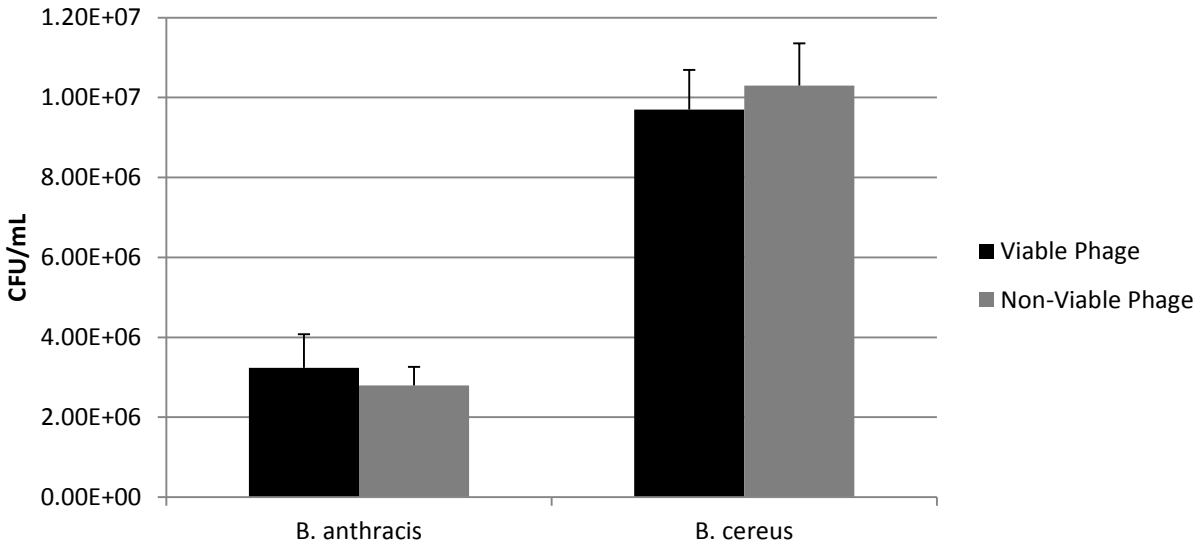

Figure 6. Viable phage do not significantly suppress bacterial growth during the first 90 minutes of exposure. Despite observing early binding to *B. anthracis* Sterne via EM (Figure 5), viable ɣ phage did not significantly alter bacterial colony counts after 90 minutes of exposure when compared to the effects of heat-inactivated phage. *B. cereus* NCTC 2599 culture counts were similarly unaffected by ɣ phage within 90 minutes of exposure.

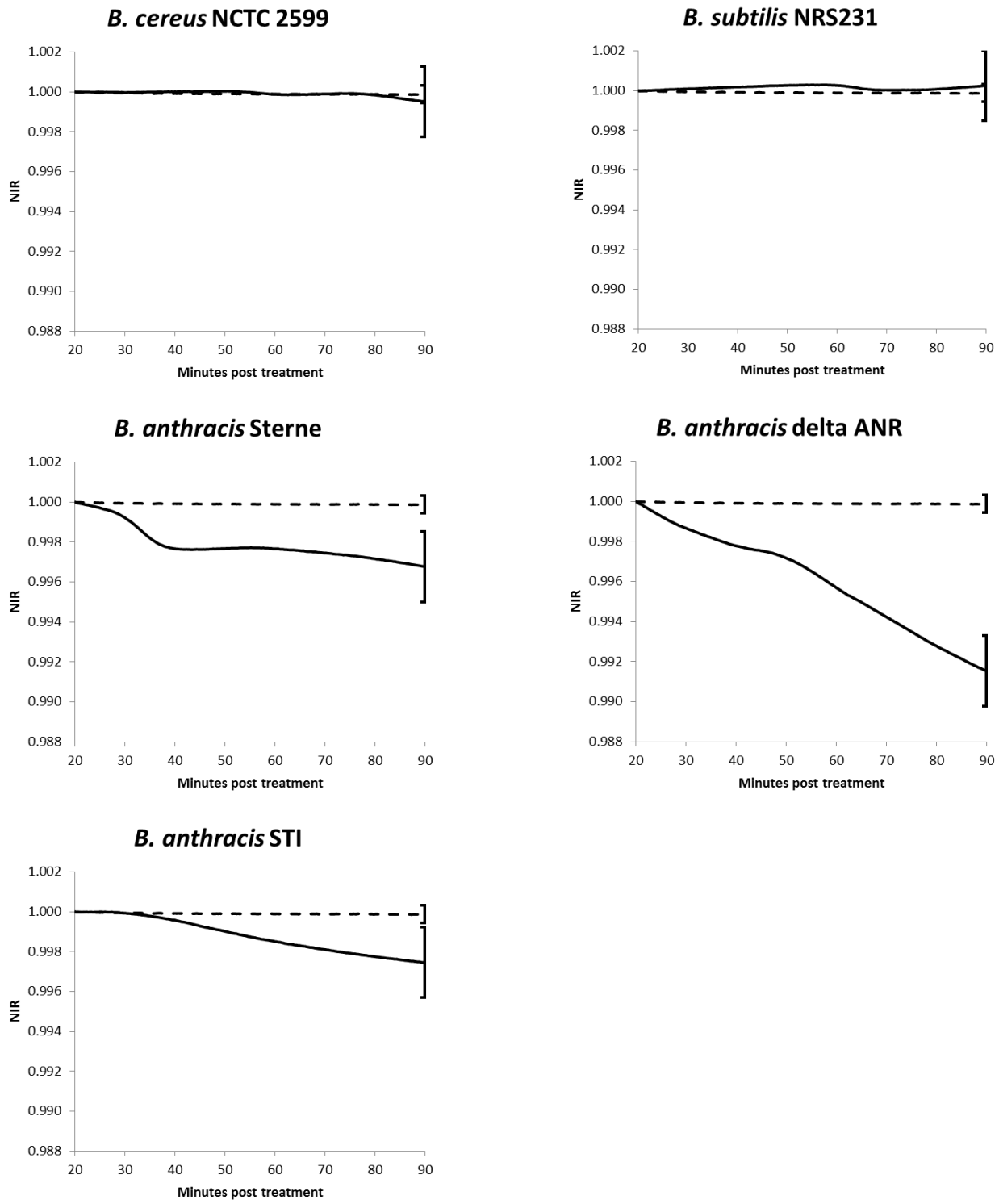

Figure 7. Ɣ phage broadly affect the normalized impedance responses for sensitive *Bacillus*. When *Bacillus* cultures are challenged with heat-inactivated and live ɣ phage in Chambers A and B, respectively, of the Z-Sense cassette, phage-sensitive *B. anthracis* exhibit a significant drop in the NIR (solid lines) when compared to runs in which only phage (without bacteria) used in both chambers (dashed lines). The NIR was calculated by dividing the capacitance of the chamber containing bacteria and viable phage with that

containing bacteria and heat-inactivated phage. Error bars depict the standard deviations obtained from three or more independent experiments.